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Inhibition of Activated Factors II, VII, IX, and X by Synthetic Organic Compounds Directed against the Active-Site Seryl Residue[1]

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Key Words. Thrombin · Factor VII_a · Factor IX_a · Factor X_a · Inhibition · Organic compounds

Abstract. A series of 53 organic chemicals belonging to the groups of organic phosphates, sulfonyl derivatives and carbamates were screened for their activity against the coagulation factors II_a (thrombin), VII_a, IX_a and X_a. Relatively specific inhibitors for the factors II_a, VII_a and X_a were found.

Introduction

In the past decade, much progress has been made in the characterization of the molecular processes underlying blood coagulation (table I). The activation reactions occurring in the coagulation pathways are highly specific, limited proteolysis reactions; the activated coagulation factors with the exception of factors V_a and $VIII_a$ belong to the class of the serine proteases. When comparing the amino acid sequences around the active-site serine residues of the coagulation factors with those of the pancreatic enzymes chymotrypsin, trypsin and elastase, it can be seen that a high degree of sequence homology exists among these proteins [4]. Furthermore, thrombin and factor X_a contain histidine and asparagin residues in positions corresponding with the active-site histidine and asparagin residues present in trypsin and chymotrypsin. In the case of thrombin and factor X_a,

[1] This work has been described in full detail in the PhD thesis of the first author (Leyden University, 1976).

Table I. A survey of the coagulation factors with proteolytic activity

Coagulation factor	Specifications of the zymogen molecule	Activation
XII	bovine factor XII: single-chain glycoprotein, molecular weight (MW) 74,000; preliminary amino acid sequence data by *Fujikawa* et al. [7, 9]; human factor XII: single-chain glycoprotein, MW 76,000 [10]	by plasma kallikrein by a single proteolytic arg-val cleavage, leading to a disulfide-linked two-chain enzyme; the in vivo initiation of the activation is not yet understood
XI	bovine factor XI: glycoprotein containing two similar disulfide-linked polypeptide chains, MW 124,000 [19]; human factor XI: glycoprotein, containing two identical disulfide-linked polypeptide chains; partial amino acid sequence known [2, 22]	by factor XII_a, in both chains a new N-terminal Ile residue is created; factor XI_a is a disulfide-linked four-chain molecule
IX	bovine factor IX: single-chain glycoprotein, MW 55,400; amino acid sequence nearing completion [39]; human factor IX: single-chain glycoprotein; MW 57,000; amino acid sequence nearly identical to that of bovine factor IX [35]	by factor XI_a ($+Ca^{++}$) in a two-step reaction; in the first step an arg-ala bond is cleaved, leading to a disulfide-linked two-chain molecule, next an activation peptide is removed from the heavy chain by arg-val cleavage
VII	bovine factor VII: single-chain glycoprotein, MW 45,500 [18, 33, 34]	by arg-ile cleavage; the in vivo initiation of the activation is not clear
X	bovine factor X: two-chain glycoprotein, MW 55,000; amino acid sequence completely known [6, 8, 15, 40]; human factor X: two-chain glycoprotein, MW 58,900 [35]	by tenase complex (IX_a, $VIII_a$, Ca^{++}, phospholipids) through arg-ile cleavage in the N-terminal region, releasing a 9,000-dalton fragment
II	bovine factor II: single-chain glycoprotein, MW 70,000; amino acid sequence completely known [24]; human factor II: single-chain glycoprotein, MW 70,000; amino acid sequence almost elucidated [3, 38]	by prothrombinase complex (X_a, V_a, Ca^{++}, phospholipids) in a two-step reaction; in the first step an arg-thr bond is cleaved; the second arg-ile cleavage yields the two-chain disulfide-linked thrombin molecule with MW 37,000

the amino acid sequence of the active-site-bearing chain shows about 40–45% sequence homology with trypsin. These data strongly suggest a close analogy between the coagulation factors and the pancreatic enzymes with respect to the activation of the zymogen and the catalytic mechanism of the active enzyme. Most likely, the substrate-binding pocket of the coagulation proteases will resemble that of trypsin because all coagulation proteases are specific towards basic amino acids (table I).

The main differences between the coagulation factors and the digestive enzymes are the presence of a second protein chain in the coagulation factors not involved in the catalytic reaction per se and, perhaps as a consequence of the occurrence of this additional protein part, the higher substrate specificity of the coagulation factors.

Of course, the tertiary structure of the coagulation factors can only be proven by crystallographic studies of these enzymes and their (natural) substrates, but valuable information can be obtained from work with model substrates. In these studies, small changes in the substrate structure can be introduced, and the effects on the enzyme-substrate interaction brought about by these small changes can give useful information about the substrate-binding site and the catalytic site of the enzymes. The same holds true for inhibitors.

The aim of the work presented here was to find specific inhibitors for the different coagulation factors. The availability of such specific inhibitors would be useful for the study of the mechanism of blood coagulation and for the chemical determination of the coagulation factors. The stereochemistry of the specific inhibitors could contribute to our knowledge of the structure of the active site of the coagulation factors, and finally, the specific inhibitors might possibly be used therapeutically. In the experiments described here, the inhibitory capacity towards the activated coagulation factors was checked for a series of organic compounds belonging to the organic phosphates, the sulfanyl derivatives and the carbamates. Other groups have used the same approach, but the families of inhibitors investigated here have, to our knowledge, not been screened before [13, 16, 30, 31].

Materials and Methods

Isolation and Purification of the Factors II_a, VII_a, IX_a and X_a [see also 40]

As starting material, a coagulation factor concentrate containing the factors II, VII, IX, and X was prepared according to *Swart* [37]. The coagulation factor concentrate was activated with thromboplastin and Ca^{2+}; the activated coagulation factors were separated

by chromatography on a Whatman DE 32 column. (For the factors II_a and VII_a the best results were obtained when the isolation was carried out at 4 °C; for factor X_a the best results were obtained when the isolation occurred at room temperature.) It was not possible to obtain factor IX_a by this procedure, presumably because the starting material did not contain a sufficient amount of this factor. However, when the coagulation factor concentrate was activated with contact product, a factor IX_a preparation could be obtained. This preparation always contained factor XI_a (from the contact product) and was only used in preliminary experiments. It was also possible to isolate a factor $VII_a - IX_a - X_a$ concentrate from serum and to isolate the factors VII_a and X_a from this concentrate by chromatography on Sephadex G-100.

No attempt was made to obtain preparations that contained clotting factors that could be considered pure by the usual physicochemical criteria. Preparations that contained one coagulation factor in excess and no or trace activities of the others were considered sufficiently pure for our purposes. This is because the inhibitors are added in large molar excess anyhow, and the tests employed are sufficiently specific not to be influenced by trace amounts of inhibited or uninhibited coagulation factors other than the one under investigation.

Coagulation Factor Reagents

These reagents were prepared according to *Koller* et al. [20] and *Loeliger and Koller* [23] (factor II); according to *Borchgrevink* et al. [1] (factor V); according to *Hemker* et al. [12] (factors VII and X); according to *Denson* [5] (factor VII/X reagent). The factor VIII and factor IX reagents were obtained from a patient with a severe deficiency of the respective coagulation factor (< 1 % activity) (440 ml blood collected in a siliconized glass vessel containing 60 ml ACD solution; storage at −20 °C). Factor XI reagent was obtained from congenital factor XI deficient plasma.

Thromboplastin was prepared from human brain according to *Owren and Aas* [32]. Contact product was prepared according to *Niewiarowski* et al. [29].

Determination of Coagulation Factors

For the estimation of the factors II, V, VII and X, one-stage estimations were carried out as described by *van der Meer* et al. [26]; the factors VIII and IX were estimated according to *Veltkamp* et al. [42]; factor XI was estimated according to *Horowitz* et al. [14].

Determination of the Activated Coagulation Factors

Factor II_a was determined according to *Hemker* et al. [11]; factor VII_a in the same system as factor VII [26] but with phospholipids instead of thromboplastin; the factors $VIII_a$ and IX_a in the same systems as used for factors VIII and IX [42] except that no kaolin was added to the test system and the incubation time of sample and reagent prior to recalcification was 1 min instead of 30 min; factor X_a was determined in a one-stage test with a factor VII and X deficient reagent and phospholipid [26].

Inhibitors were the same as described by *Meyers* [27] and *Meyers* et al. [28].

Inhibition Experiments

The coagulation factor (concentration about 10^{-6}–10^{-7} mol/l) was incubated with the inhibitor (concentration about 10^{-2}–10^{-4} mol/l) at pH 7.4, 37 °C, in siliconized glass or in plastic during 30 min (the concentrations of the coagulation factors were: thrombin,

12.5 NIH U/ml; factor VII_a, 0.4–0.6 U/ml; factor IX_a, 0.15–0.25 U/ml; factor X_a, 0.5–0.75 U/ml; the final inhibitor concentration is given in tables II–V. If there was no or only a slight inhibition after 30 min, the enzymatic activity was tested again after 1, 2, 3 or, sometimes, 18 h.

All chemicals were p.a. grade from Merck.

Protein concentrations were determined by measuring $E_{280\,nm}$ in a Zeiss PM Q 11 spectrophotometer. It was assumed that for prothrombin and thrombin, 1 extinction unit at 280 nm corresponds with 1.6 mg protein/ml [17, 37] and for all other protein mixtures with 1 mg protein/ml [36].

For a number of compounds the nuclear magnetic resonance (NMR) spectra were recorded in a Jeol PS 100 NMR apparatus; sweep time 250 s, sweep width 1,080 Hz ($\times$ 0.01 ppm). The organic compounds were dissolved in $CDCl_3$; with the compounds No. 1, 7, 13, and 30, a little deuterated aceton was added.

Results and Discussion

Within a series of related inhibitors that are all able to react with an active-site serine residue, the actual course of the reaction is determined by the stereochemistry of the enzyme around the active-site residue [21]. Therefore, it can be expected that the differences between the coagulation factors will be reflected in their susceptibility towards related inhibitors with varying chemical structure. As far as small substrates are considered, this is the same phenomenon that accounts for substrate specificity. For this reason, the interaction of series of serine esterase inhibitors with the factors II_a, VII_a, IX_a, and X_a has been investigated.

The evaluation of the kinetic constants of the inhibition process was based on the following general scheme for the reaction of serine proteases with irreversible inhibitors.

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot I \xrightarrow{k_{+2}} E_{inhibited} + P$$

For this situation the steady-state approximation yields

$$\frac{d[E \cdot I]}{dt} = k_{+1}([E][I] - K_i[E \cdot I]) = 0$$

with

$$K_i = \frac{k_{-1} + k_{+2}}{k_{+1}}$$

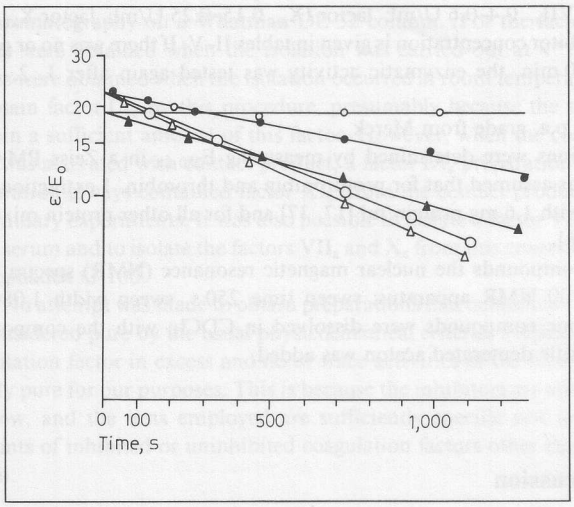


Fig. 1. Time course of the inhibition of thrombin by dinitrophenyl-benzyl-phosphate in the following concentrations: ○ = blank; ● = 0.08 mmol; ★ = 0.19 mmol; ◯ = 0.31 mmol; ☆ = 0.39 mmol.

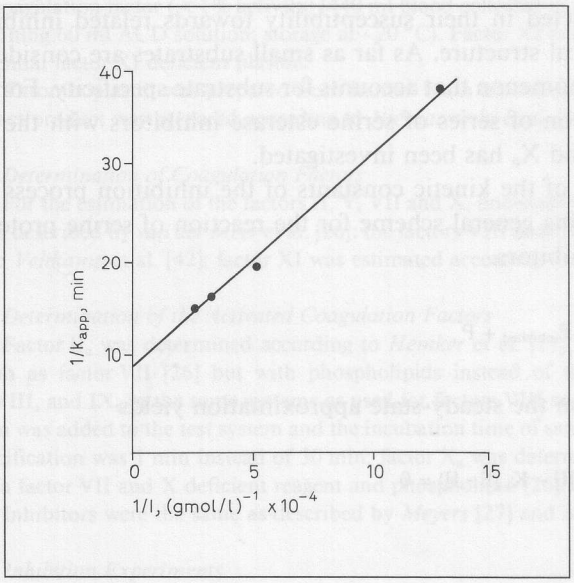


Fig. 2. The dependence of k_{app} on the concentration of dinitrophenyl-benzyl-phosphate.

If $[E_{akt}]$ stands for $[E] + [E \cdot I]$, the total amount of uninhibited enzyme, it can be deduced that the formation of the irreversibly inhibited enzyme proceeds with the velocity

$$v = \frac{-d[E_{akt}]}{dt} = k_{+2} \frac{[I]}{[I] + [K_i]} [E_{akt}].$$

Integration of this differential equation yields

$$[E_{akt}]_t = [E_{akt}]_0 \exp\left(-k_{+2} \frac{[I]}{[I] + K_i} t\right).$$

When the residual activity ($[E_{akt}]_t/[E_{akt}]_0$) after the incubation time t is plotted on semilogarithmic paper against the incubation time, a straight line is obtained. The slope of this line gives the apparent reaction constant (k_{app}) of the inactivation process. In formula:

$$k_{app} = -k_{+2} \frac{[I]}{[I] + K_i}.$$

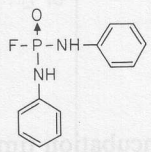
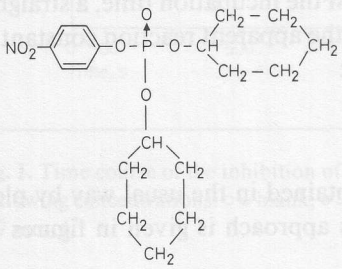
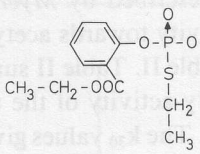
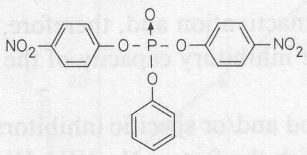
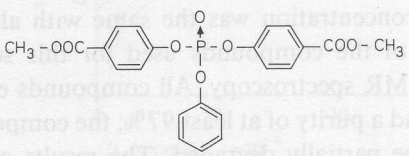
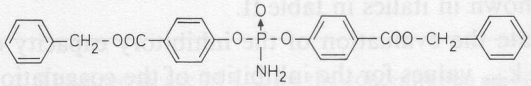
The values of k_{+2} and K_i can be obtained in the usual way by plotting $1/k_{app}$ against $1/[I]$. An example of this approach is given in figures 1 and 2.

The inhibitors used in this study were first described by *Myers* and co-workers [26, 27] who tested their inhibitory activity towards acetylcholine esterases; their chemical structure is given in table II. Table II summarizes the results of a first screening of the inhibitory activity of the tested compounds towards the factors II_a, VII_a, IX_a and X_a. The k_{30} values given in table II were calculated after a fixed constant time interval (30 min) without a control of the actual time course of the inactivation and, therefore, must be considered as a rough estimation of the inhibitory capacity of the compounds under consideration.

The compounds that seemed to be good and/or specific inhibitors during this first screening were tested again with the factors II_a, VII_a, IX_a, and X_a, but this time the inhibitor concentration was the same with all four coagulation factors. The purity of the compounds used for this second screening was also checked by NMR spectroscopy. All compounds except compounds No. 13, 16, and 35 had a purity of at least 97%; the compounds No. 13, 16, and 35 seemed to be partially degraded. The results of this second screening are shown in italics in table II.

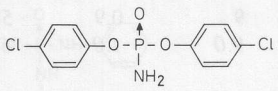
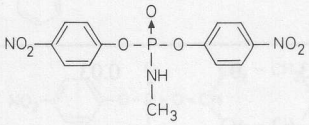
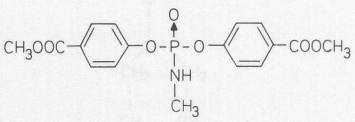
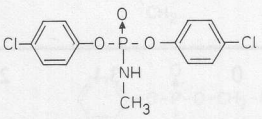
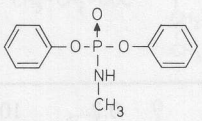
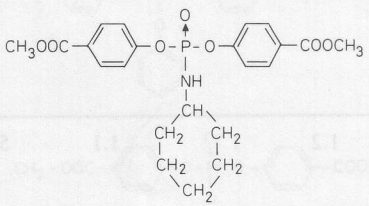
In order to facilitate the evaluation of the inhibitory capacity of the compounds tested, the k_{app} values for the inhibition of the coagulation factors with diisopropylfluorophosphate (DFP) are given in table III.

Table II

No.	Chemical structure of the inhibitor
1.	
2.	
3.	
4.	
5.	
6.	

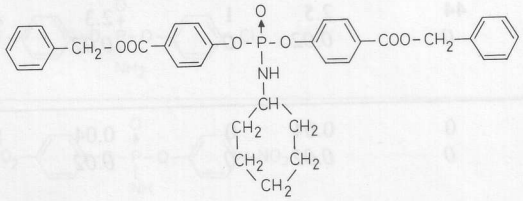
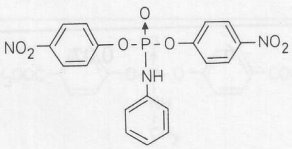
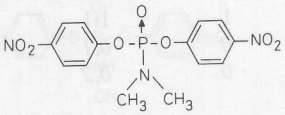
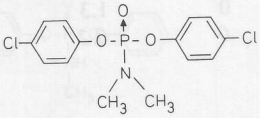
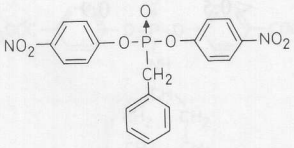
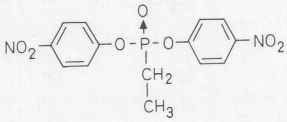
II_a inhibition		VII_a inhibition		IX_a inhibition		X_a inhibition	
$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}	$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}	$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}	$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}
0.5	15	1.0	0	1.0	9	0.9	58
1.0	*20*	*1.0*	*0*	*1.0*	*9.0*	*1.0*	*60*
0.03	3	0.03	0	0.03	0	0.03	0
3.1	0	1.7	16	3.1	0	3.1	20
0.1	> 128	0.1	70	2.6	3	0.2	105
0.04	*> 128*	*0.04*	*20*	*0.04*	*0*	*0.04*	*2.9*
0.6	> 128	0.6	44	1.2	1	1.1	58
0.02	89	0.02	< 6	4.04	1	0.04	24
0.02	*89.4*	*0.02*	*< 5.9*	*0.02*	*0*	*0.02*	*15.9*

Table II (cont.)

No.	Chemical structure of the inhibitor
7.	
8.	
9.	
10.	
11.	
12.	

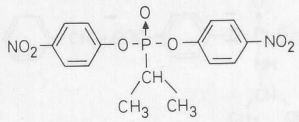
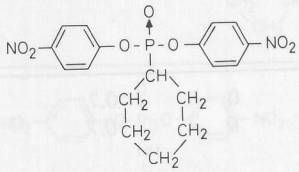
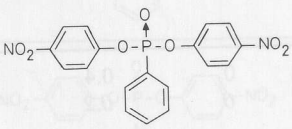
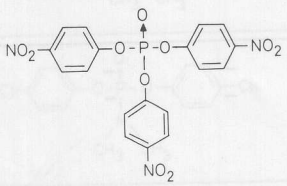
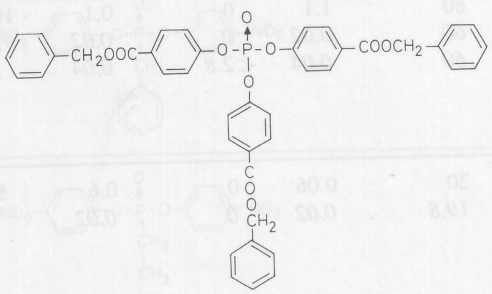
II_a inhibition		VII_a inhibition		IX_a inhibition		X_a inhibition	
$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}	$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}	$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}	$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}
1.3	> 128	1.3	44	2.5	1	2.3	40
0.02	*> 128*	*0.02*	*0*	*0.02*	*0*	*0.02*	*0*
0.02	44	0.02	0	0.04	0	0.04	12
0.02	*50.9*	*0.02*	*0*	*0.02*	*0*	*0.02*	*0*
0.04	20	0.04	16	0.08	6	0.07	16
0.06	12	0.6	< 3	1.4	1	1.1	12
0.1	*0*	*0.1*	*0*	*0.1*	*0*	*0.1*	*0*
0.2	*9.0*	*0.2*	*0*	*0.2*	*0*	*0.2*	*0*
0.6	12	0.6	< 3	1.3	0	1.3	0
0.2	*5.9*	*0.2*	*0*	*0.2*	*0*	*0.2*	*0*
0.5	6	0.5	20	0.8	< 0.5	0.9	6

Table II (cont.)

No.	Chemical structure of the inhibitor
13.	
14.	
15.	
16.	
17.	
18.	

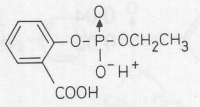
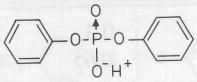
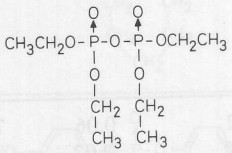
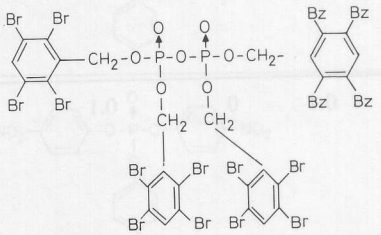
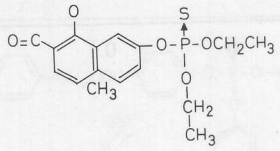
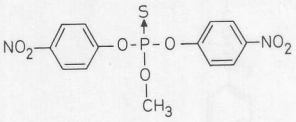
II_a inhibition		VII_a inhibition		IX_a inhibition		X_a inhibition	
$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}	$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}	$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}	$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}
–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–
0.1	12	0.9	0	0.8	0	0.7	6
0.2	*23.9*	*0.2*	*0*	*0.2*	*0*	*0.7*	*0*
0.2	20	0.2	0	0.2	0	0.4	6
0.2	*20*	*0.2*	*2.8*	*0.2*	*0*	*0.2*	*2.8*
–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–
0.05	> 128	0.05	60	1.1	0	0.1	105
0.02	*> 128*	*0.02*	*60*	*0.02*	*0*	*0.02*	*28.4*
0.04	*> 128*	*0.04*	*60*	*0.04*	*< 2.8*	*0.04*	*38.5*
0.03	> 128	0.03	20	0.06	0	0.6	58
0.02	*> 128*	*0.02*	*19.8*	*0.02*	*0*	*0.02*	*0*

Table II (cont.)

No.	Chemical structure of the inhibitor
19.	
20.	
21.	
22.	
23.	

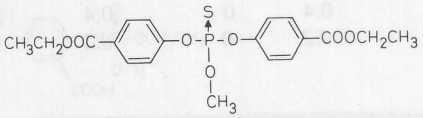
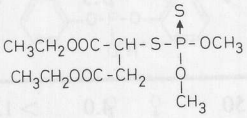
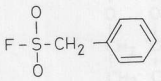
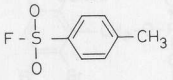
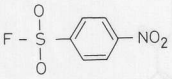
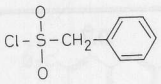
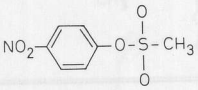
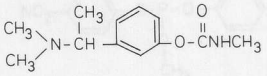
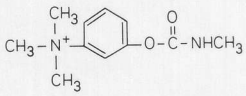
II_a inhibition		VII_a inhibition		IX_a inhibition		X_a inhibition	
$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}	$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}	$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}	$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}
0.02	> 128	0.04	10	0.04	0	0.04	33
0.02	> 128	0.02	5.9	0.02	0	0.02	< 5.9
0.1	> 128	0.4	0	0.4	0	0.3	28
0.05	*58.3*	*0.05*	*0*	*0.05*	*0*	*0.05*	*0*
0.04	> 128	0.04	128	0.8	12	0.06	77
0.02	*> 128*	*0.02*	*44.4*	*0.02*	*0*	*0.02*	*9.0*
0.08	20	0.08	16	0.1	0	0.1	0
0.1	6	0.1	0	0.1	0	0.1	0

Table II (cont.)

No.	Chemical structure of the inhibitor
24.	
25.	
26.	
27.	
28.	
29.	

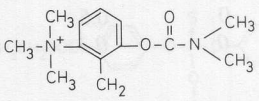
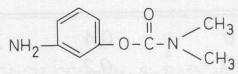
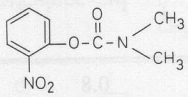
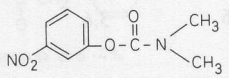
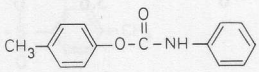
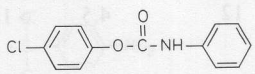
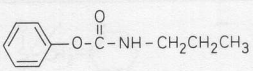
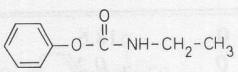
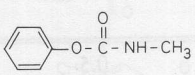
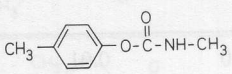
II_a inhibition		VII_a inhibition		IX_a inhibition		X_a inhibition	
[I][a] mmol/l	k_{30} 10^{-4} min^{-1}	[I][a] mmol/l	k_{30} 10^{-4} min^{-1}	[I][a] mmol/l	k_{30} 10^{-4} min^{-1}	[I][a] mmol/l	k_{30} 10^{-4} min^{-1}
0.2	16	0.2	33	0.4	0	0.4	20
0.2	*16.0*	*0.2*	*33.2*	*0.2*	*0*	*0.2*	*15*
0.1	20	7.3	0	7.5	5	7.5	40
2.0	> 128	2.0	40	4.0	50	4.0	> 128
0.003	< 3	0.003	6	0.2	6	0.006	12
0.4	16	0.7	0	0.7	0	0.7	6
0.6	0	0.6	3	0.6	0	1.1	13

Table II (cont.)

No.	Chemical structure of the inhibitor
30.	
31.	
32.	 isoprop. a.d. 4%
33.	
34.	
35.	
36.	
37.	
38.	

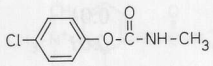
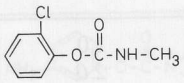
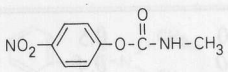
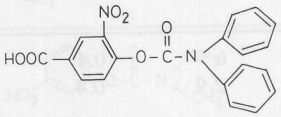
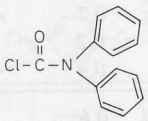
II_a inhibition		VII_a inhibition		IX_a inhibition		X_a inhibition	
[I][a] mmol/l	k_{30} 10^{-4} min^{-1}	[I][a] mmol/l	k_{30} 10^{-4} min^{-1}	[I][a] mmol/l	k_{30} 10^{-4} min^{-1}	[I][a] mmol/l	k_{30} 10^{-4} min^{-1}
0.3	> 128	0.3	20	0.9	0	0.5	20
0.3	*> 128*	*0.3*	*19.8*	*0.3*	*0*	*0.3*	*15*
0.2	< 9	0.2	77	0.2	0	0.4	20
0.2	*9.0*	*0.2*	*77*	*0.2*	*0*	*0.2*	*5.9*
0.8	24	0.8	0	0.8	0	0.8	0
2.0	28	2.0	0	3.6	6	3.6	77
2.0	128	2.0	128	4.0	0	3.6	20
2.5	> 128	2.5	77	8.0	12	4.5	> 128
–	–	–	–	–	–	–	–
0.2	6	0.2	9	18.6	0	18.6	0
0.2	*5.9*	*0.2*	*9.0*	*0.2*	*0*	*0.2*	*0*
0.3	20	0.3	12	0.5	0	0.5	0
0.2	*2.9*	*0.2*	*0*	*0.2*	*0*	*0.2*	*0*
0.01	10	0.01	0	0.01	0	0.01	0
0.01	*10*	*0.01*	*0*	0.01	*0*	*0.01*	*0*

Table II (cont.)

No.	Chemical structure of the inhibitor
39.	
40.	
41.	
42.	
43.	
44.	
45.	
46.	
47.	
48.	

II_a inhibition		VII_a inhibition		IX_a inhibition		X_a inhibition	
[I][a] mmol/l	k_{30} 10^{-4} min^{-1}	[I][a] mmol/l	k_{30} 10^{-4} min^{-1}	[I][a] mmol/l	k_{30} 10^{-4} min^{-1}	[I][a] mmol/l	k_{30} 10^{-4} min^{-1}
0.01	12	0.01	0	0.01	0	0.01	*0*
0.01	*12.4*	*0.01*	*0*	*0.01*	*0*	*0.01*	*0*
1.0	6	1.0	6	1.0	0	1.0	0
2.0	*23.9*	*2.0*	*0*	*2.0*	*0*	*2.0*	*0*
0.3	3	0.3	3	0.5	0	0.5	12
0.4	*0*	*0.4*	*0*	*0.4*	*0*	*0.4*	*0*
0.2	9	0.2	3	0.4	6	0.4	0
0.4	*16.0*	*0.4*	*0*	*0.4*	*2.9*	*0.4*	*3.9*
0.4	20	0.4	33	1.7	0	0.7	<6
0.2	*16.0*	*0.2*	*2.9*	*0.2*	*0*	*0.2*	*0*
0.4	*19.8*	*0.4*	*33.0*	*0.4*	*0*	*0.4*	*<2.9*
0.4	6	0.4	6	0.6	0	0.6	0
0.6	*19.8*	*0.6*	*9.0*	*0.6*	*0*	*0.6*	*<2.9*
2.5	9	2.5	44	5.1	3	4.6	20
0.5	*5.9*	*0.5*	*0*	*0.5*	*0*	*0.5*	*5.9*
3.2	0	1.6	44	3.2	0	2.8	20
0.5	*0*	*0.5*	*0*	*0.5*	*0*	*0.5*	*9.0*
0.8	0	0.4	44	0.8	0	1.4	6
0.2	*0*	*0.2*	*0*	*0.2*	*0*	*0.2*	*12*
1.0	*0*	*1.0*	*20*	*1.0*	*16*	*1.0*	*0*
0.3	3	0.3	3	0.6	12	0.6	12
0.4	*2.8*	*0.4*	*0*	*0.4*	*1.9*	*0.4*	*12*
0.2	*0*	*0.2*	*0*	*0.2*	*1.9*	*0.2*	*12*

Table II (cont.)

No.	Chemical structure of the inhibitor
49.	
50.	
51.	
52.	 Tris-HCl buffer
53.	 alc. Tris buffer 4%

* The in vitro inhibitory activity of the $(R)_3 - O - P \rightarrow S$ type compounds is mainly due to the $(R)_3 - S - P \rightarrow O$ isomers, which are present as an impurity in the preparations in equilibrium with the former compounds.

II_a inhibition		VII_a inhibition		IX_a inhibition		X_a inhibition	
$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}	$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}	$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}	$[I]^a$ mmol/l	k_{30} 10^{-4} min^{-1}
0.2	9	0.2	0	0.2	0	0.2	6
0.2	*9.0*	*0.2*	*0*	*0.2*	*0*	*0.2*	*12*
0.2	< 3	0.2	< 3	0.2	0	0.2	10
0.2	*< 2.8*	*0.2*	*< 2.8*	*0.2*	*0*	*0.2*	*12*
0.2	< 20	0.2	< 6	0.2	0	0.6	< 6
0.2	*19.8*	*0.2*	*2.9*	*0.2*	*0*	*0.2*	*2.9*
2.4	> 128	2.4	105	4.3	6	4.3	128
0.2	*33.2*	*0.2*	*19.8*	*0.2*	*0*	*0.2*	*0*
0.5	22	0.5	12	0.8	20	0.8	77
0.5	*22.2*	*0.5*	*11.7*	*0.5*	*19.8*	*0.5*	*28*

Table III. Inhibition of the coagulation factors II_a, VII_a, IX_a, and X_a by DFP

Coagulation factor	[DFP] mmol/l	k_{app} 10^{-4} min^{-1}
II_a	0.2	3,600
VII_a	6.9	60
IX_a	4.2	
X_a	50	300

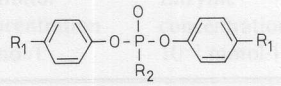
As judged from table II, there seem to occur rather specific inhibitors for the different coagulation factors among the organic compounds tested. The inhibitors that gave k_{30} values of 50×10^{-4} min^{-1} in these screenings were used further to determine the k_{+2} and K_i values of the inactivation processes (see below). As the data on the ineffective inhibitors gave information on the active site as well, we did not omit the negative results.

Tables IV–VI give the kinetic constants of the interaction of the factors II_a, VII_a, and X_a, respectively, with their more or less specific inhibitors. For the determination of the k_{+2} and K_i values given in these tables, samples were taken from the incubation mixture at different times and the residual activity was measured. k_{+2} and K_i values were calculated as described above. By sampling from the incubation mixture and dilution with the substrate, the velocity of the inhibition reaction decreases and competition between the inhibitor and natural substrate occurs. Because of this dilution, and because the clotting times are short compared to the incubation times, it can be assumed that the inactivation reaction does not proceed further during the clotting test. For the same reasons, the inhibition of thromboplastin and of the coagulation factors in the reagent by the inhibitor during the activity determination can be neglected.

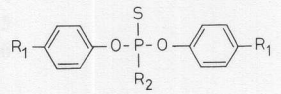
As can be deduced from table II, among the rather specific irreversible thrombin inhibitors, compounds No. 7 and 19 are the most promising. Furthermore, it can be seen in this table that compound No. 31 inhibits preferentially factor VII_a and compound No. 1 inhibits preferentially factor X_a. Compound No. 17 inhibits strongly all three factors, thrombin, VII_a and X_a. Of course, it should be kept in mind that table II shows the results of a rough first screening and gives no real kinetic constants.

When comparing the tables IV, V, and VI, it is striking that the 'specific' inhibitors all belong to the class of the organic phosphorus com-

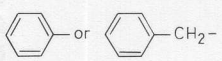
pounds. Moreover, all the irreversible thrombin inhibitors except compound No. 30 have the structure:



while compound No. 30 has the structure:



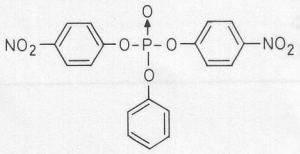
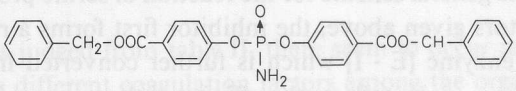
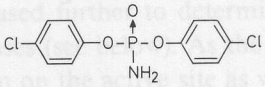
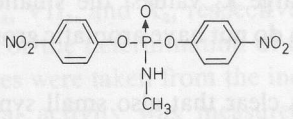
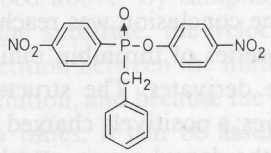
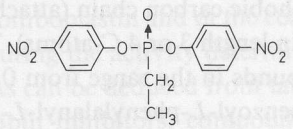
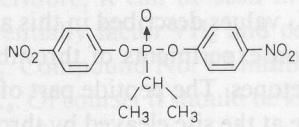
As can be seen in the general scheme for the reaction of serine proteases with irreversible inhibitors given above, the inhibitor first forms a reversible complex with the enzyme [E · I] which is further converted into an irreversible complex [$E_{inhibited}$]. K_I reflects the affinity of the enzyme for the substrate, and k_2 is the reaction velocity of the irreversible step. As can be seen in table IV, the compounds with a



group in the R_2 position give relatively large k_2 values; the smallest K_I values are found with the compounds which do not have aromatic groups in the R_2 portion.

From the results presented above it is clear that also small synthetic compounds can discriminate between the closely related compounds thrombin, factor VII_a and factor X_a. The same conclusion was reached by other groups. *Okamoto* et al. [30] studied a series of thrombin inhibitors belonging to N^{α}-naphtalenesulfonyl-*L*-argine derivates. The structure of these inhibitors is characterized by three entities: a positively charged group (the amino acid argine), an aromatic group (the dansyl group, attached to the α-NH_2 group of arginine), and a hydrophobic carbon chain (attached to the carboxyl group of arginine; optimal chain length 3 or 4 C-atoms). These authors reported I_{50} values for these compounds in the range from 0.03 to 2 µmol/l when fibrinogen (3 µmol/l) or N^{α}-benzoyl-*L*-phenylalanyl-*L*-valyl-*L*-arginine-*p*-nitroanilide (100 µmol/l) is used as a substrate. These values are of the same order of magnitude as the K_I values described in this article. *Kettner and Shaw* [16] reported inactivation experiments of thrombin by tetra- and tripeptide argine chloromethyl ketones. The peptide part of these substrates contains the amino acid sequence at the site cleaved by thrombin in its physiological substrates. The compounds Val-Pro-Arg-CH_2Cl (analog of the factor XIII cleavage site) and Ile-Pro-Arg-CH_2Cl (analog of the pro-

Table IV. Specific inhibition of factor II_a

No.	Chemical structure of the inhibitor
4.	
6.	
7.	
8.	
17.	
18.	
19.	

Inhibitor concentration mmol/l	Enzyme concentration 10^{-3} mmol/l	k_{app} 10^{-2} min^{-1}	k_2 10^{-2} min^{-1}	K_i 10^{-5} mol/l
1.30	1.6	2.60	3.1	20
1.04		2.62		
0.65		2.42		
0.26		1.77		
0.20	2.3	2.21	2.5	5.3
0.16		1.80		
0.10		1.65		
0.04		1.16		
1.24	1.7	2.36	2.8	34
0.99		1.97		
0.62		1.30		
0.25		1.25		
0.47	4	3.68	4.9	15
0.28		3.10		
0.14		2.39		
0.56	4	23.1	25	22
0.39		28.9		
0.28		11.6		
0.33	4	3.03	4	7.9
0.33		3.47		
0.20		1.62		
0.10		2.17		
2.00	1.7	2.41	3	100
1.60		1.40		
1.00		1.23		
0.40		1.04		

Table IV. (cont.)

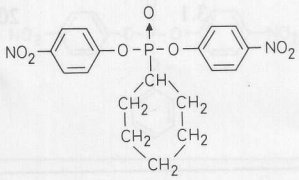
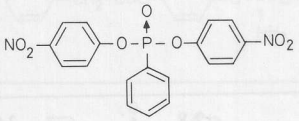
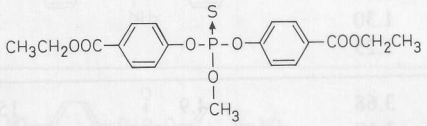
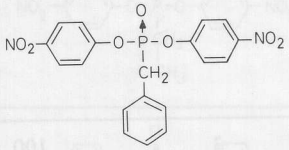
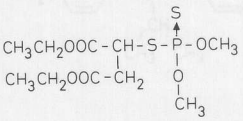
No.	Chemical structure of the inhibitor
20.	
21.	
30.	

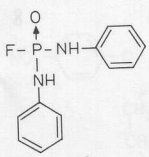
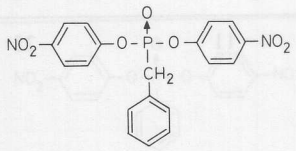
Table V. Specific inhibition of factor VII_a

No.	Chemical structure of the inhibitor
17.	
31.	

Inhibitor concentration mmol/l	Enzyme Concentration 10^{-3} mmol/l	k_{app} 10^{-2} min^{-1}	k_2 10^{-2} min^{-1}	K_i 10^{-5} mol/l
1.63	2.3	2.29	2.5	8
1.31		2.13		
0.82		2.64		
0.33		2.00		
0.39	2.3	6.71	11	25
0.31		6.12		
0.19		5.20		
0.08		2.65		
0.44	2.3	2.05	2.5	10
0.35		1.96		
0.22		1.73		
0.09		1.16		

Inhibitor concentration mmol/l	Enzyme concentration 10^{-3} mmol/l	k_{app} 10^{-2} min^{-1}	k_2 10^{-2} min^{-1}	K_i 10^{-5} mol/l
1.11	3.6	3.30	calculation impossible	
0.89		3.15		
0.55		3.78		
0.22		9.04		
22.8	3.6	9.2	25	2,500
18.2		13.9		
18.2		16.0		
11.4		11.2		
4.6		3.1		

Table VI. Specific inhibition of factor X_a

No.	Chemical structure of the inhibitor
1.	
17.	

thrombin cleavage site) inactivate thrombin by 50% in less than 25 min at a concentration of 7.5×10^{-8} *M*. The compound Gly-Val-Arg-CH_2Cl [analog of the cleavage site in the fibrinogen A (α) chain] was effective at a concentration of 2×10^{-6} mol/l. The authors reported that the compounds with the proline residue in the P_2 composition were markedly more reactive with thrombin than with plasma kallikrein, plasmin, urokinase, and factor X_a. *Markwardt* [25] described irreversible thrombin inhibitors derived from the competitive inhibitors benzylamine and benzamidine by introduction of a reactive fluorosulfonyl moiety at the aromatic ring. These compounds, aminomethyl and amidinofluorosulfonylbenzenes, possessed an inhibitory effect towards thrombin that surpassed that of DFP and phenylmethyl sulfonylfluoride (PMSF).

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Inhibitor concentration mmol/l	Enzyme concentration 10^{-3} mmol/l	k_{app} 10^{-2} min^{-1}	k_2 10^{-2} min^{-1}	K_i 10^{-5} mol/l
0.97	0.25	5.7	20	200
0.78		4.8		
0.49		3.2		
0.19		2.0		
1.11	0.25	7.3	7.7	27.6
0.88		5.1		
0.55		4.9		
0.22		3.4		

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